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13. ABSTRACT *(Maximum 200 Words)*

An enhanced growth of FGF treated estrogen receptor positive (ER+) breast cancer cells in medium containing an antiestrogen ICI 182,780 suggests that FGF signaling completely bypasses a requirement for ERα activation for growth. A lipid-anchored docking protein SNT-1 links FGFR molecules with the Ras/MAP kinase signaling pathway. The main objective of this work is the validation of SNT-1 as a useful target for drug development. For this purpose we cloned a phosphotyrosine binding domain (PTB) of SNT-1 in an expression vector and stably transfected a human ER+ breast cancer cells to study the possible dominant negative effect of the PTB domain on FGF dependent growth phenotype. Using semiquantitative RT PCR we demonstrated different levels of mRNA expression in different transfected clones and studied the FGF-dependent growth of the clones expressing higher levels of the PTB domain. We developed a colony forming test in FGF-dependent growth conditions to study a possible dominant negative effect of the SNT-1 PTB domain. A mammalian two-hybrid system which we developed lacked the sensitivity for fine mapping of the SNT-1 PTB domain interaction with the FGFR juxtamembrane domain. We overexpressed the PTB domain of SNT-1 in E.coli and purified a protein to be used in the Biacore system and for crystallization experiments to study the PTB domain structure and its interaction with FGFR1,2,3,4 peptides.

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Introduction

Approximately 50-70% of breast cancer patients with estrogen receptor positive tumors who fail on either primary of adjuvant tamoxifen therapy have tumors that still express estrogen receptor (ER) (1). Growth factor signaling can provide ER positive breast cancer cells with an alternative growth stimulus to that which is provided by activation of ER. Previous studies from many laboratories have demonstrated that FGFs and FGF receptors are expressed in human breast tumors (2, 3). Data from the laboratory of principal investigator indicate that FGF-1 and FGF-4 transfected MCF-7 cells become tamoxifen resistant and cross resistant to second line antihormonal agents including pure antiestrogen ICI 182,780 (Faslodex) and two aromatase inhibitors (4). An enhanced growth of FGF-transfected or FGF-treated ERα+ breast cancer cells in medium containing ICI 182,780, an antiestrogen which greatly increases the rate of ER degradation, suggests that FGF signaling completely bypasses a requirement for ERα activation for growth. The RAS/MAPK pathway plays an important role in signaling via FGF receptors. Published work from other laboratories has indicated that a lipid-anchored docking protein called SNT-1 links FGFR molecules with the Ras/MAP kinase signaling pathway (5). SNT-1 protein contains a phosphotyrosine binding (PTB) domain at its amino terminus. This PTB domain binds to a juxtamembrane region of FGFR1 (6). Several proteins, including Grb2/Sos, Shp2, Crk, Gab1, Src, PKC ζ, and PKC λ bind to SNTs following FGF induced SNT phosphorylation (7, 8, 9). Because FGF is a growth factor that may have paracrine as well as autocrine effects within a tumor, including effects on angiogenesis, interference with this binding may also block the growth promotion that occurs in endothelial cells or other stromal elements. The three objectives of this project included: 1) validation of SNT-1 as a useful target for drug development; 2) definition of the minimal region of FGFR-1 and the other three FGFRs that can interact with SNT-1; and 3) determining a crystal structure of the SNT-1 PTB domain.

Body

Determining the ability of the PTB domain of SNT-1 to function as a dominant negative inhibitor of FGF signaling in ER+ breast cancer cells.

We constructed an expression vector using the pCMVTag vector from Stratagene which contained an RT-PCR generated cDNA of the N terminal 140 amino acids of the SNT-1 protein (including the myristylation signal and the phosphotyrosine binding domain) in front of a c-myc epitope. ML20 breast cancer cells were cotransfected with this vector and the vector pEF6 conferring resistance to blasticidin. We initially used polyclonal transfected cell lysates for Western blotting with a c-myc antibody to identify protein expression. Although the antibody showed a positive reaction with a control protein (Positop) it did not identify a c-myc tagged PTB domain in cellular lysates. It could be that the sensitivity of the antibody used for Western blot (5 ng) is lower than necessary to identify the level of PTB domain expression in this polyclonal population. We plan to concentrate protein by immunoprecipitation prior to Western blotting. To avoid difficulties of protein expression determination in polyclonal populations, we also used a clonal approach. Clonal populations of blasticidin resistant cells were isolated, expanded, and assayed for expression of SNT-1 mRNA by semi-quantitative RT PCR using Access RT-PCR System (Promega). Since the PTB domain cDNA was cloned between the BamHI and XhoI sites of the pCMVTag vector, it was therefore flanked by T3 and T7 promoters. In order to avoid detection of a PTB domain PCR product from the chromosomal copy of SNT-1, we designed two sets of RT-PCR primers. In one set the upstream primer was located at the 5' end of the cloned gene and the downstream primer in the vector T7 promoter sequence. In the other set one primer was located in the T3 promoter sequence and another at the 3' end of the PTB sequence. In each reaction we used the primers that would detect an 804 b.p. fragment of the housekeeping gene GAPDH as an internal control for the RT PCR reaction. We identified different levels of PTB domain mRNA expressed in different clones (Fig. 1). We could expect that the growth of transfected ML20 clones expressing higher levels of PTB domain mRNA would no longer exhibit FGF-dependent growth phenotypes due to the dominant negative effect of PTB domain on SNT-1 function. We used a growth curve experiments to check this possibility. Cells were cultured for 3 days

in estrogen-deprived conditions and plated in 24-well dishes at 20,000 cells per dish. The next day cells were exposed to the following growth conditions: Improved Minimal Essential Zinc Option medium (Gibco BRL) supplemented with 5% fetal bovine serum (5% FBS IMEM); 5% FBS IMEM + 10^{-7}M of the antiestrogen ICI 182,780(ICI); 5% FBS IMEM+ICI+10 ng/ml of FGF1, 5%FBS IMEM+ICI+10^{-7} M of 17β–estradiol(E2). Cells were counted on 3rd, 5th, and 7th days of incubation. As a result of this experiment we found one clone (clone 9) which showed an inhibition of FGF-dependent growth in ICI 182,780 containing media as compared to ML20 cells transfected with vector alone or to the other clones (Fig. 2). To demonstrate a correlation between levels of growth inhibition and levels of PTB protein expression, we plan to determine the SNT-1 PTB domain protein levels in the clones expressing different levels of PTB mRNA by immunoprecipitation with antibodies to FRS2 followed by Western blotting with c-myc antibodies. We also started looking for reduced tyrosine phosphorylation of wild type SNT-1 protein in these clones in response to FGF stimulation. We checked for wild type SNT-1 phosphorylation in the absence of FGF and after FGF1 treatment for different periods of time. We used pSuc13 conjugated agarose to precipitate SNT-1 with subsequent Western blot with anti-phosphotyrosine antibodies. We were able to identify phosphorylated SNT-1 after stimulation with FGF1 in the parental ML20 cell line. We plan to look for a reduction of wild type SNT-1 phosphorylation in different clones transfected with SNT-1 PTB domain in response to FGF treatment.

Study of SNT-1 - FGFR1 interaction in vivo: mammalian two-hybrid system.

To determine the minimal interacting region within the FGFR juxtamembrane region that is capable of interacting with the SNT-1 PTB domain and to create a system for screening of drugs inhibiting an interaction between SNT-1 and FGF receptors, we utilized a mammalian two-hybrid assay (Clontech). The PTB domain and juxtamembrane domain of FGFR1 each were cloned separately in the pBIND vector as fusion proteins with a GAL4 DNA binding domain and the vector pACT as fusion proteins with the herpes simplex virus VP16 activation domain. ML20, Cos-7, and NIH3T3 cells were transiently cotransfected with the newly constructed plasmids and the reporter plasmid pG5Luc which contains five GAL4 binding sites upstream of a minimal TATA box locate is upstream of the firefly luciferase gene. As a result

of these experiments we found that the PTB domain of SNT-1 and the juxtamembrane domain of FGFR1 can interact but this interaction is very weak compared to two positive control interacting fusion proteins, Gal4-Id and MyoD-VP16 provided in the kit. Consequently, we have abandoned this approach. Furthermore, as discussed below, the need to define minimal interacting domain is now less pressing in light of the recent publication of Ong S.H. et al. who used site-directed mutagenesis to determine the important amino residues involved in the interaction (10).

Determining a crystal structure of the phosphotyrosine binding domain of SNT-1 protein.

Two recent reports support in essence the novel aspects of the SNT-1 PTB domain FGFR1 juxtamembrane region interaction originally suggested by the homology model we developed using the structure of IRS-1 PTB domain as a template. This model suggested a separate binding domain for the basic FGFR peptide residues within an acidic groove of the SNT-1 PTB domain that was distinct from the phosphotyrosine binding domain. In our model we suggested that residues 401-435 of FGFR1 would be important for the complex formation with PTB domain of SNT-1. Recent literature data supported this suggestion. The importance of residues 412-433 of FGFR1 in the complex formation with the PTB domain of SNT-1 was recently demonstrated by mutational analysis and coimmunoprecipitation (10). Moreover, as suggested in our model, the basic residues 418-427 were determined to be needed for this interaction. NMR analysis (11) suggested that FGFR1 residues 411-430 interact with a separate domain that is distinct form the phosphotyrosine binding site and involves residues 18-116. NMR coordinates will be available in June, 2001 and will be used as a basis for further comparison.

Since the information about the mode of interaction of PTB domain of SNT-1 with the other members of FGFR family is not yet available, we will attempt to study a possible interaction of the PTB domain with each of the FGFR2, FGFR3, FGFR4 peptides homologues to the FGFR1 interacting region using a Biacore system (Biacore) and through cocrystallization with purified PTB domain. These data will provide the precise structural data necessary for efficient drug design.

The SNT-1 PTB cDNA in the pCMVTag vector was used as a template for PCR reactions with primers that resulted in the separate amplification of subregions of the PTB domain of SNT-1 protein (residues 11-140, 11-125, 11-106). These cDNAs were engineered to lack the myristylation signal but were cloned in frame with an N-terminal 6His-Tag that is part of the pET15b expression vector (Novagen) and used to transform the host strain E.coli BL21-Codon Plus(DE3)-RIL. Recombinant protein synthesis was induced by addition of 1mM Isopropyl β-Thiogalactopyranoside (IPTG) into the growth media at 37^0C during exponential growth. *E. coli* cells transformed with plasmids containing the SNT-1 PTB domain (residues 11-140 and 11-125) overproduced recombinant proteins containing 6His-Tag at their N-termini. Most of the overexpressed proteins were found in the "inclusion bodies". Since native protein would be preferred for the protein crystallization, we continued working on protein purification from the soluble fraction but the yield ultimately proved to be insufficient for purification purposes. Our efforts to localize more protein in the soluble fraction instead of inclusion bodies by modification of growth conditions or IPTG concentrations did not significantly change protein distribution between cellular fractions. Therefore, it was decided to purify the proteins from "inclusion bodies" with subsequent renaturation. The purification procedure was as follows. The frozen cell pellet from 1 liter of *E.coli* culture was resuspended in lysis buffer (20 mM sodium phosphate, pH7.4, 500 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, and protease inhibitors), and extracted by two passes through French press. After 30 min. centrifugation at 27,000g the pellet was washed twice with lysis buffer and resuspended overnight in lysis buffer containing 6 M guanidine-HCl. The solution was clarified by a second centrifugation and applied to a Ni^{2+}-agarose cartridge (Pharmacia) equilibrated with 10 mM imidazole, 400 mM NaCl, 20 mM sodium phosphate, pH 7.4-7.6, and 6 M guanidine-HCl. The column was washed with equilibration buffer containing 60 mM imidazole and the recombinant SNT1-PTB domain was eluted from the column with 5 ml of equilibration buffer containing 500 mM imidazole. The eluate was refolded overnight by drop-wise dilution (~15 fold) into 25 mM Tris•HCl, pH 8.0, 500 mM KCl, 1 mM DTT, and 10% glycerol (refolding buffer). The refolded SNT1-PTB domain was loaded overnight onto a second Ni^{2+}–agarose column equilibrated with refolding buffer. The column was washed with 30 ml of refolding buffer containing 10 mM imidazole and the protein eluted with 30 ml of buffer containing 500 mM imidazole. The solution was

immediately passed over a Sephadex G-25 column equilibrated with 25 mM Tris•HCl, pH 8.0, 500 mM KCl, 1 mM DTT, and 0.1 mM EDTA. The *N*-terminal His_6-Tag was removed by overnight digestion on ice with thrombin (Sigma T-3010; 2 U/ml); digestion was halted by elution through a benzamidine-agarose column. The sample was then applied to a preparative gel filtration column (Pharmacia HiLoad 26/60 Superdex 200 prepgrade) equilibrated with 25 mM Tris•HCl, pH 8.0, 500 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol. The majority of protein eluted from the column in a single peak corresponding to a molecular weight of 14,000 daltons. The fractions from the peak which contained a single protein were pooled, concentrated (Millipore BioMax 15-10,000) to ~10 mg/ml in 25 mM Tris•HCl, pH 8.0, 150 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol, and stored at -80 °C. A typical yield from a 1 Liter of *E. coli* culture was 60 mg of SNT1-PTB domain. Fig. 3 demonstrates the protein purification results.

To study the specificity and mechanism of interaction between the SNT-1 PTB domain and the juxtamembrane domains of FGF receptors, we synthesized minimum interaction peptides containing conserved amino acid sequences of FGFR1 (amino acids 411-430, Genbank accession # M34185), FGFR2 (amino acids 412-431, Genbank accession # NM_000141), FGFR3 (amino acids 408-427, Genbank accession #M58051), and FGFR4 (amino acids 403-422, Genbank accession # Y113901) juxtamembrane domains. Attempts of the crystallization procedure are in progress.

Determining a crystal structure of the phosphotyrosine binding domain of SNT-1 protein.

In collaboration with Dr. Shaomeng Wang of Georgetown University, we used the homology model we developed for pharmacophore screening of chemical structure databases. We received from Dr. Wang about 60 compounds selected by his computer assisted drug design program for their potential ability to mimic the structure of the FGFR1 peptide interacting with the acidic groove of the SNT-1 protein. The compound search was based on the homology model we developed using the PTB domain of IRS-1 protein as a template. If effective, these compounds would be expected to selectively inhibit FGF-dependent cell growth phenotypes. We looked for abrogation of FGF-dependent growth of ML20 cells in the presence of drugs in growth curve experiments. Cells were incubated in the medium deprived

of estrogen for 3 days prior to the experiment and plated in 24-well dishes at 20,000 cells/dish. Compounds were dissolved in 0.1% DMSO. Each compound was added to the cell cultures at the concentration of 100 μM. Cells were incubated in the presence of compounds for 5 days in each of the following conditions: 5% FBS IMEM, 5% FBS IMEM+ICI, 5% FBS IMEM+ ICI+FGF1, and 5% FBS IMEM+ICI+E2. We did not see a significant inhibition of FGF-dependent cell growth in the presence of these compounds (Fig. 4). Some compounds showed an additive effect with ICI, which may need future investigation to check their possible antiestrogenic effect. Since the drug design was based on the computer modeling of the target protein, it may be useful to continue the search for new peptidomimetics when the crystal structure of SNT-1 has been resolved which would increase the precision of drug design.

Key research accomplishments:

- Different levels of the SNT-1 PTB domain mRNA were identified in the stably transfected clones of ML20 cells. One clone showed inhibition of FGF-dependent growth in ICI 182,780 containing media.
- Ultimately a mammalian two-hybrid system was developed which proved the lack of sensitivity required for fine mapping of SNT-1 interacting amino acids with FGFR juxtamembrane region.
- Colony forming assays in FGF-dependent growth conditions were developed to study a possible dominant negative effect of the SNT-1 protein PTB domain.
- The PTB domain of SNT-1 was overexpressed in *E.coli* and purified to be used in the Biacore system and in the crystallization experiments to study the PTB domain structure and its interaction with FGFR1, 2, 3, 4 peptides.

Conclusions

At the end of the first year of our study, we have completed several goals listed in Tasks 1, 2, 3. We constructed a SNT-1 PTB domain expression vector and isolated clonal and

polyclonal populations of stably transfected ML20 cells and have characterized the levels of PTB domain mRNA expression in different clones. We will continue working on determining the levels and function of PTB domain protein present in these clones. To characterize a potential dominant negative effect of SNT-1 PTB domain, we will use a colony forming assay in the presence of ICI 182,780 and FGF1. We already developed a procedure for this assay in our laboratory that involves high efficiency transfection of parental ML20 breast cancer cells with an expression vector encoding both blasticidin resistance and a dominant negative signaling molecule using Lipofectamine Plus Reagent (GibcoBRL). We have already cloned the SNT-1 PTB domain into the pEF6 blasticidin resistance vector to use in these type of studies. We also started to characterize the effects of PTB domain expression on wild type SNT-1 tyrosine phosphorylation.

Study of the SNT-1 PTB domain interaction with the juxtamembrane domain of FGFR1 in the mammalian two-hybrid system partially fulfils the goals in the Task 2. Since the interaction which we demonstrated is relatively low, we are looking for a different approach involving a Biacore apparatus as an alternative method for determining an interaction of the PTB domain with FGFR2, 3, and 4 juxtamembrane peptides.

Completing a significant part of the goals in Task 3 allowed us to start establishing cocrystallization of the SNT-1 PTB domain with the minimal interaction FGFR peptides. Solving a structure of PTB domain will improve our computer assisted design of peptidomimetics abrogating FGF signal transduction.

Based on the recent literature data, another potential signaling adapter, Shc, binds to the nerve growth factor receptor, Trk, at the same phosphotyrosine residue as FRS-2 (12). Even though targets for FRS-2 protein on FGFR1 and TrkA are distinct (10), it would be interesting to study signaling through Shc as another potential link to the Ras protein in the FGF pathway in breast cancer cells.

References

1. Johnston, S.R., Saccani-Jotti, G ., Smith, I.E., Salter, J., Newby, J., Coppen, M., Ebbs, S.R., and Dowsett, M. Changes in estrogen receptor, progesterone receptor, and pS2

expression in tamoxifen resistant human breast cancer. Cancer Res., 55: 3331-3338, 1995.

2. Yoshimura, N., Sano, H., Hashiramoto, A., Yamada, R., Nakajima, H., Kondo, M., and Oka, T. The expression and localization of fibroblast growth factor-1 (FGF-1) and FGF receptor-1 (FGFR-1) in human breast cancer. Clin. Immunol. Immunopathol., 89: 28-34, 1998.
3. Anandappa, S.Y., Winstanley, J.H., Leinster, S., Green, B., Rudland, P.S., and Barraclough, R. Comparative expression of fibroblast growth factor mRNAs in benigh and malignanat breast disease. Br. J. Cancer, 69: 772-776, 1994.
4. McLeskey, S,W., Zhang ,L., El-Asjry, D., Trock, B.J., Lopez, C.A., Kharbanda, S., Tobias, C.A., Lorant, L.A., Hannum, R.S., Dickson, R.B., and Kern, F.G. Tamoxifen-resistant FGF-transfected MCF-7 cells are cross-resistant in vivo to the antiestrogen, ICI 182,780 and two aromatase inhibitors. Clin. Cancer Res. 4:697-711, 1998.
5. Kouhara, H., Hadari, Y.R., Spivak-Kroizman,T., Schillling, J., Lax, I., and Schlessinger, J. A lipid-anchored Grb2 binding protein that links FGF receptor activation to the Ras/MAP kinase signaling pathway. Cell, 89:693-702, 1997.
6. Xu, H., Lee, K.W., and Goldfarb, M. Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. J. Biol.Chem., 273:17987-17990, 1998.
7. Hadari, Y.R., Kouhara, H., Lax I., Schlessinger, J. Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. Mol. and Cell. Biol., 18:3966-3973, 1998.
8. Larsson, H., Klint, P., Landgreen, E., Claesson-Welsh, L. Fibroblast growth factor receptor-1-mediated endothelial cell proliferation is dependent on the Src homology (SH) 2/SH3 domain-containing adaptor protein Crk. J. of Biol. Chem., 274:25736-25734, 1999.
9. Lim, Y.P., Low, B.C., Lim, J., Wong, S.M., Guy, G.R. Association o atypical protein kinase C isotypes with the docker protein FRS2 in fibroblast growth factor signaling. J. of Biol. Chem., 274:19025-19034, 1999.
10. Ong S.H., Guy, G.R., Hadari, Y.R., Laks, S., Gotoh, N., Schlessinger, J., Lax, I. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on

fibroblast growth factor and nerve growth factor receptors. Mol.and Cell. Biol., 20: 979-989, 2000.

11. Dhalluin, C., Yan, K.S., Plotnikova, O., Lee, K.W., Zeng, L., Kuti, M., Mujtaba, S.,Goldfarb, M., Zhou, M.-M. Structural basis of SNT PTB domain interactions with distinct neurotropic receptors. Molecular Cell, 6: 921-929, 2000.
12. Meakin, S.O., MacDonald, J.I.S., Gryz, E.A., Kubu, C.J., Verdi, J.M. The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. J. of Biol. Chem., 274: 9861-9870, 1999.

Appendix

Figures and legends

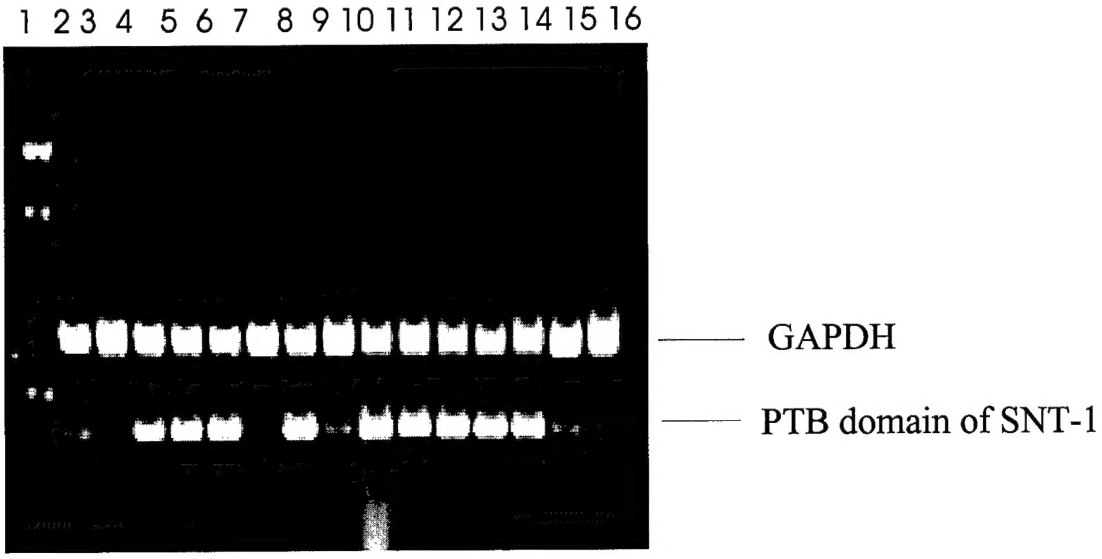

Figure 1. RT PCR of SNT-1 PTB domain in different ML20 clones.

Lane 1. 100 b.p. DNA markers (Gobco BRL).
Lanes 2-15. RT PCR of the PTB domain from different ML20 clones stably transfected with pCMVTag/PTB expression vector
Lane 16. RT PCR of the PTB domain from the ML20 clone stably transfected with pCMVTag vector.

The upstream primer used for PTB domain RT PCR amplification contained the same 5'-sequence (5'-CCCGGGGACACTGTCCCAGATAACC-3') that was used to PCR amplify the PTB domain. The downstream primer was located within T7 promoter sequence of vector pCMVTag.

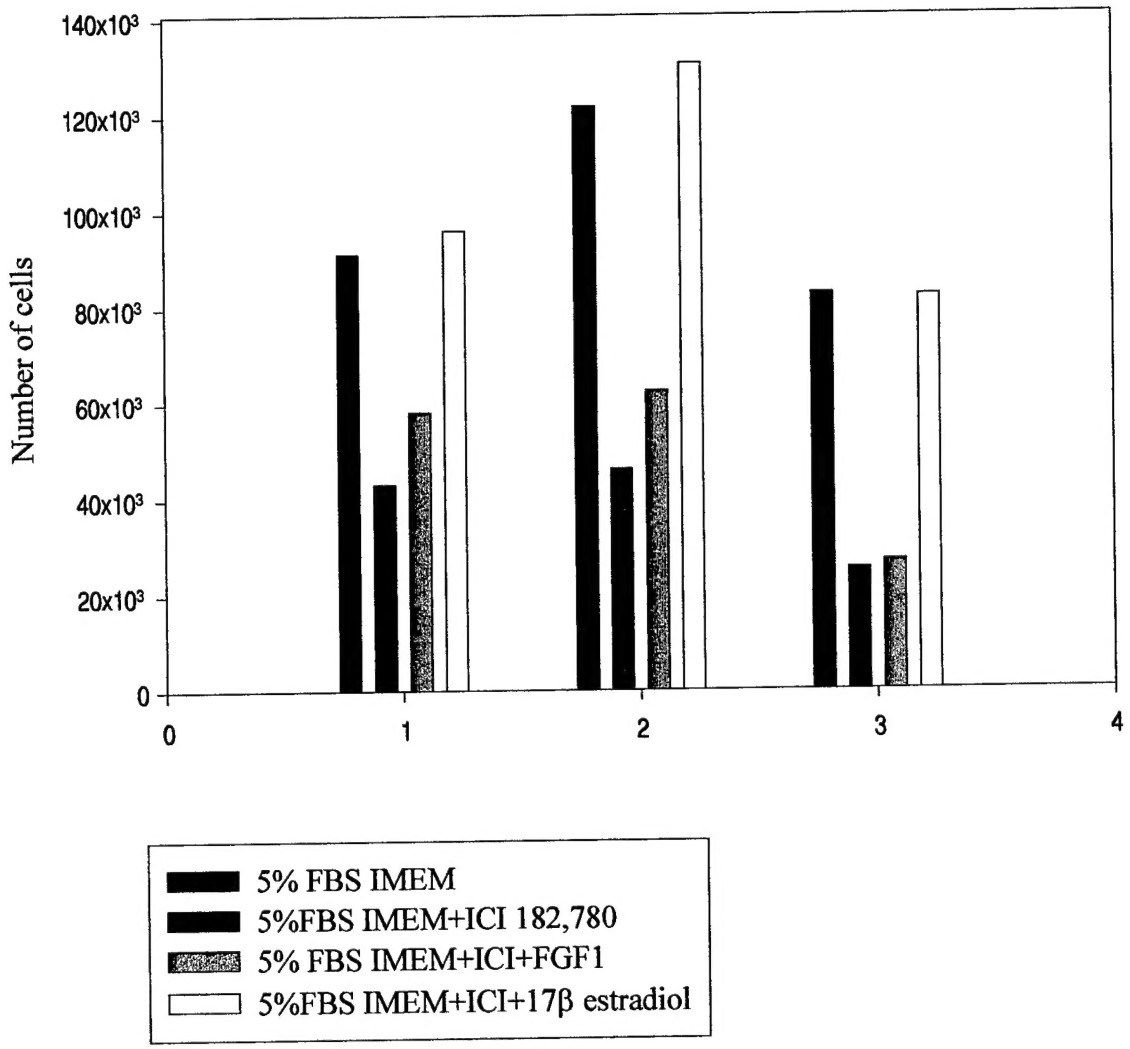

Figure 2. Inhibition of FGF dependent growth of ML20 cells stably transfected with pCMVTag/PTB domain expression vector (clone 9). Cells were stripped of estrogen for 3 days and plated in 24-well dishes at 20,000 cells per dish. 24 hours later (Day 0) media were replaced with 5% FBS IMEM or 5% FBS IMEM containing different ingredients. Antiestrogen ICI 182,780 and 17β estradiol were used at 10^{-7}M, FGF1 at 20 ng/ml. Media were replaced every 48 hours. Cell counts are shown for the Day 7.

Bar group 1. ML20 cells.
Bar group 2. ML20 cells transfected with vector pCMVTag.
Bar group 3. ML20 cells transfected with expression vector pCMVTag/PTB (clone 9).

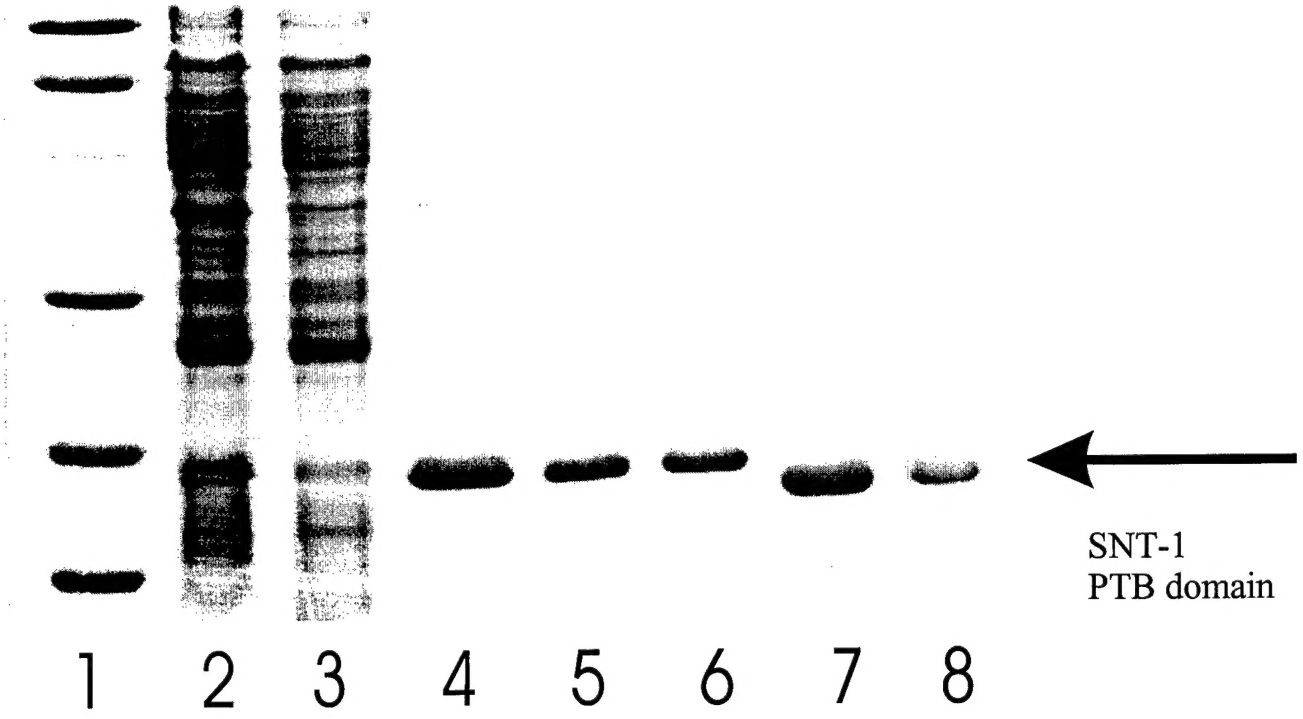

Figure 3. **Purification of SNT-1 PTB domain from *E.coli* BL21-Codon Plus (DE3)RIL**

Lane 1. Molecular weight standards (BioRad).
Lane 2. Total *E.coli* cell lysate.
Lane 3. Supernatant of lysed *E.coli* cells.
Lane 4. Pellet of *E.coli* cells dissolved in the lysis buffer with 6M guanidine HCl.
Lane 5. Eluate of Ni column(wash with the buffer containing 60 mM of imidazole).
Lane 6. PTB domain refolded from Ni eluate.
Lane 7. PTB domain after digestion with thrombin.
Lane 8. PTB domain after purification by the gel filtration on Pharmacia HiLoad 26/60 Superdex 200 column

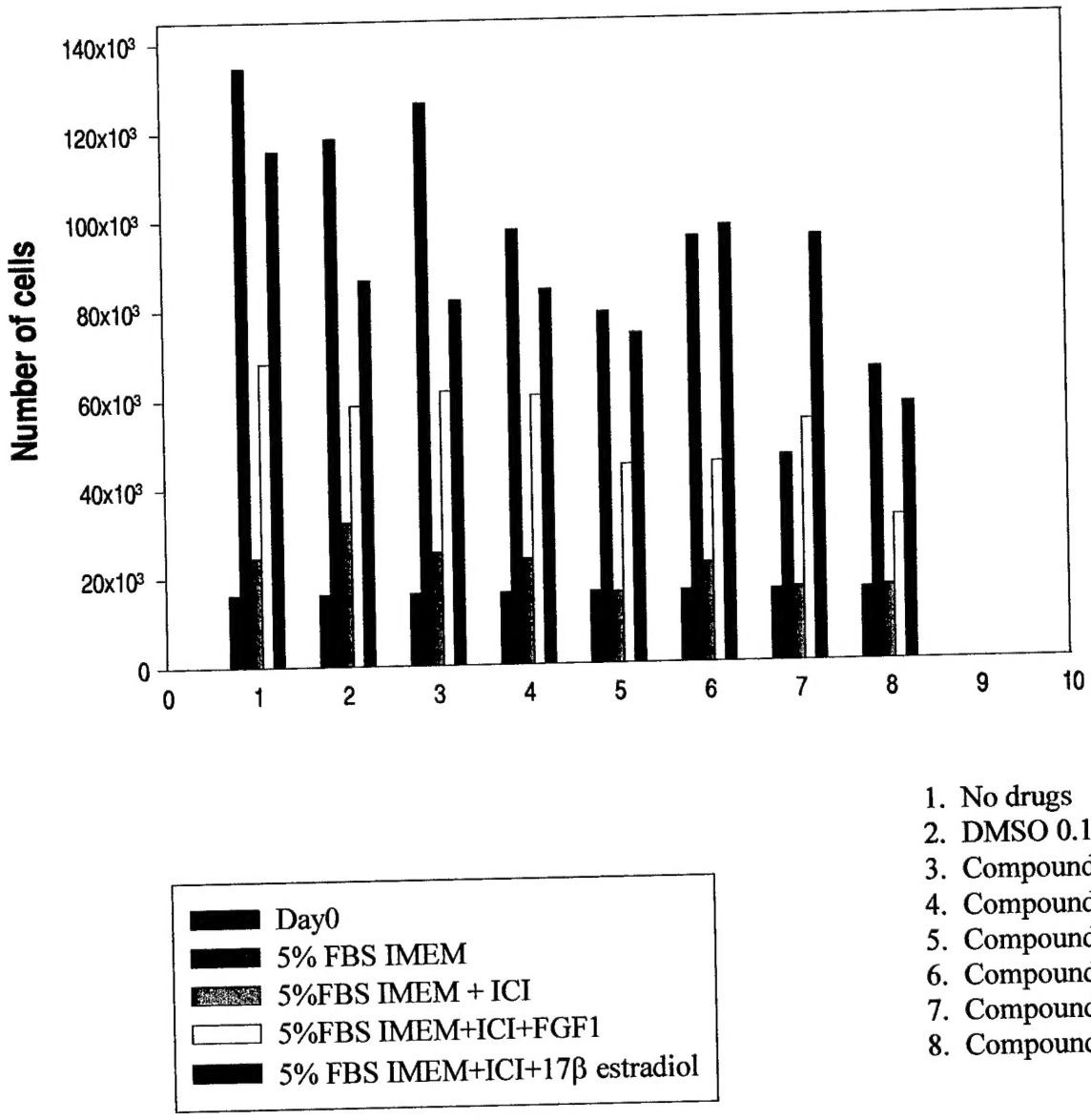

Figure 4. Influence of potential anti-SNT-1 peptido-mimetics on the growth of ML20 cells. ML20 cells were stripped of estrogen and plated in 24-well dishes at 20,000 cells per dish in the estrogen-free media. 24 hours later (Day0) media were replaced with 5%FBS IMEM or 5%FBS IMEM containing different ingredients. Antiestrogen ICI 182,780 and 17β estradiol were used at 10^{-7}M, FGF1 at 20 ng/ml. Media were replaced every 48 hours. Cells were counted on the 7th day after addition of compounds.